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Summary

In the present study the processes of the formation of necrotic lesions on cowpea leaves infected with cucumber mosaic virus were investigated in relation to two basic characteristics : hypersensitive reaction and oxidation of phenols.

No significant changes were found in the polyphenol oxidase, peroxidase and phenylalanine ammonia-lyase activities or in the contents of phenolic compounds before a significant morphologic manifestation of local lesion. The activities of peroxidase and phenylalanine ammoni-lyase increase after necrotic lesions appear, but polyphenol oxidase activity remains unchanged during the development of the local lesions. On the other hand, both lipoxygenase activity and respiration increase in the period prior to the appearance of lesions. Furthermore, the formation of hypersensitive lesions is markedly prevented by the addition of lipid anitoxidants which are the inhibitors of lipoxygenase, but is only slightly suppressed by the addition of inhibitors of Cu- and Fe-containing enzymes. From these results, it is thought that the oxidation of phenolic compounds is not involved in the hypersensitive reaction in the primary infected cells. It is postulated that the hypersensitive reaction evoked by viral infection in copwea leaves is caused by the activation of some oxidiative reactions in which lipoxygenase may play an important role as a catalyzer.

Of the biochemical processes relevant to the hypersensitive reaction of virus infected host tissue, the polyphenol-polyphenolase system has received most attention, recently (3, 9, 18, 20, 22). Some workers (5, 6, 23), however, believe that other factors rather than toxic substances derived from phenolics are important for the hypersensitive reaction in local lesion hosts. Indeed, Weintraub and Ragetli (23) were unable to detect any significant change in the phenolic compounds in the necrotic lesions produced by tobacco mosaic virus (TMV) in the leaves of *Nicotiana glutinosa*. Gill (6), working with TMV-infected bean, reproted similar results.

On the other hand, Yoshii (25) found that the necrotic lesion of *Nicotiana glutinosa* by TMV-infection is entirely different in its nature from the necrotic lesion of hypersensitive wheat against the invasion of an incompatible race of the

obligate parasite, *Puccinia graminis*.

Understanding the biochemical and the physiological changes that occur in the lesion formation process in plants infected by virus may provide a key to understanding this hypersensitive reaction.

The purpose of the present study was to determine if polyphenol oxidase, peroxidase and phenolic compounds play any role in the hypersensitive reaction evoked by a cucumber mosaic virus infection in cowpea leaves.

Materials and Methods

Test plant: Cowpea plants (*Vigna sinensis* Endl. *var. sesquipedalis*, cultivar. Kurodane-sanjaku) which were grown at 25°C in a green house were used at the primary leaf stage.

Virus: The virus used in the experiment was the yellow strain of cucumber mosaic virus (CMV). CMV was purified by the method of Scott (15). The upper surface of one primary leaf of cowpea plant was inoculated with CMV solution by rubbing with carborandum. The opposite leaf which was treated by inoculation with a phosphate buffer solution (0.1 M, pH 7.0) served as a control.

Samples for analysis: Since it was difficult to isolated the lesion area from the inoculated area, heavily inoculated whole leaf whose surface was over 50 per cent covered with local lesions was used in the present study. Inoculum was used in a high concentration of CMV in order to obtain 1,000 or more lesions per inoculated leaf. Visible local lesions appeared within about 20 hr.

Measurement of polyphenol oxidase and peroxidase activity: Crude enzyme solution was prepared as follows; After a certain period of inoculation, the inoculated leaves were homogenized in the cold with 0.1 M Tris-HCl buffer (pH 7.5). Then the homogenates were centrifuged at 10,000 $\times$g for 15 min at 4°C. The supernatant solution was used for the assay of enzyme activity.

Peroxidase activity was measured by the method of Farkas *et al.* (4). The complete system for the peroxidase assay consisted of 10 μM of guaiacol, 20 μM of hydrogen peroxide, enzyme solution diluted to give a liner reaction rate for at least 2 min, and 0.1 M Tris-HCl buffer (pH 7.5) in 3.0 ml final volume. The enzyme activity was expressed as the change in optical density under the conditions described above at 500 nm during 1 min.

The peroxidase activity was also detected histochemically by the *in vivo* application of a 1 per cent benzidine solution-3 per cent H_2O_2. Samples were taken from small pieces of both uninfected and CMV-infected cowpea leaves at different stages of infection. Each of the samples from the infected leaves contained a portion of one or more lesions.

The polyphenol oxidase activity was determined according to the amount of dark-colored polymeric compounds formed from chlorogenic acid. The reaction mixture contained 10^{-2} M chlorogenic acid solution, 5×5^{-2} M sodium sulfanilate

(pH 5.5) and enzyme solution. The enzyme activity was expressed as the change in optical density under the conditions described above at 500 nm during 1 min (16).

Measurement of lipoxygenase activity: For the assay of lipoxygenase activity the method of Holden (7) was used with a slight modification as shown below. The enzyme solution from cowpea leaves was prepared by centrifuging the homogenate at 5,000 ×g for 10 min. The pellet after the centrifugation was washed once with an acetate buffer (0.2 M, pH 6.0) and then suspended in a small volume of the same buffer, and this suspension was used as the enzyme solution. The 25 ml of reaction mixture which was incubated at 21°C contained 5 mg of linoleic acid in 0.5 ml ethanol, acetate buffer (4×10^{-2} M, pH 6.0), and the enzyme solution. After 15 min, the reaction was stopped by the addition of absolute ethanol containing 1 per cent HCl. The enzyme activity was determined by optical density at 560 nm.

Determination of total polyphenolic compounds and flavonol contents: Total polyphenolic compounds in infected and uninfected cowpea leaves were extracted as follows; Ten grams of infected and uninfected cowpea leaves were extracted with 70 per cent methanol at 100°C for 3 min. The methanol extract obtained was then concentrated to 1 ml under reduced pressure and the polyphenolic compounds of the concentrated solution were determined by the method of Arnown (1).

The flavonol in infected and uninfected cowpea leaves was extracted according to the method of Kuwatsuka (10). Two grams of cowpea leaves were extracted with 70 per cent methanol at 100°C for 3 min and the residue was extracted three times by 70 per cent methanol. To 1 ml of methanol extract was added 1 ml of Na_2CO_3 saturated with 70 per cent methanol plus 8 ml of 70 per cent methanol. Then the optical density of this mixture at 400 nm (εNa) was measured. To the other 1 ml of methanol extract was added 9 ml of 70 per cent methanol and the optical density at 400 nm (ε) was measured. The flavonol content of the cowpea leaves was calculated by subtracting ε from εNa.

Histochemical detection of phenolic compounds: The detection of phenolic compounds was carried out by observing the reactions to diazotized sulfanilic acid and ferric chloride in samples from infected and uninfected leaves.

Paper chromatography of phenolic compounds: For the preparation of phenolic compounds the method of Linkens (12) was used. The sample solution was spotted onto the starting point of the filter-paper (Toyo Roshi No. 51) and developed by the ascending method of two-dimensional chromatography. First direction: n-butanol - acetic acid - water (100 : 27 : 73 v/v/v); second direction: 2 per cent aqueous acetic acid. The spots of phenolic compounds on the chromatograms were examined by ultraviolet light and diazotized sulfanilic acid, followed by treatment with ammonia fumes and 0.1 per cent $FeCl_3$ solution.

Detection of quinone: All experimental procedures used for the detection of quinone were similar to those described by Farkas *et al.* (3).

Measurement of phenylalanine ammonia-lyase activity: Samples for measurement of enzyme activity were excised from infected leaves successively after the inoculation. The preparation of the enzyme solution was made by the method of Minamikawa and Uritani (14). Enzyme activity was assayed using a modified version of the method of Koukol and Conn (11). The reaction mixture consisted of 20 μM of L-phenylalanine, 100 μM of sodium borate buffer (pH 8.8) and enzyme solution with a total volume of 2.0 ml. It was incubated for 2 hr at 37°C in a water bath. The reaction was then stopped with 10 N H_2SO_4. The acidified solution was extracted twice with ethyl ether. The combined ether layers were extracted with 0.05 M NaOH and the absorbancy at 268 nm was measured.

Determination of protein concentration: The protein concentration was determined according to the method of Lowry *et al.* (13) using bovine serum albumin as a reference standard.

Respiration: The respiration of cowpea leaves was assayed manometrically by a Warburg respirometer in leaf disks obtained by the method previously described (21).

All experiments have been repeated at least three times. Averages or representative examples are given in the tables and figures.

Results

Time course formation of necrotic lesion: The time course formation of necrotic lesions in CMV-infected cowpea as observed by light microscope was as follows: the collapse of epidermal cells and the shriveling of mesophyll cells were typical reactions in the primary infected cells at about 10–11 hr after inoculation under this experimental condition. At this time, no necrosis was apparent. Browning of the cell wall of the mesophyll cell just beneath the collapsed epidermis occurred about 15 hr later. Then, the chloroplasts of these mesophyll cells disintegrated but the nucleus preserved its integrity. Successively, the browning granules appeared in the cytoplasms of the mesophyll cells, and finally the infected mesophyll cells entered the state of necrobiosis, so-called hypersensitive death. Necrosis of the primary infected epidermal cells occurred after the necrosis of the underlying mesophyll cells. Thereafter, browning spread occasionally to the tissues around the initial necrotic cells thus enlargeing the lesion size.

Changes in enzyme activity: It seemed worth while to note whether or not the increase in enzymatic activity in local lesion host preceded the hypersenitive death of the infected cells or visible lesion formation. Experiments were conducted with CMV-infected cowpea leaves within 30 hr after infection. The results for polyphenol oxidase are shown in Fig. 1. There was no significant change in the polyphenol oxidase activity before and after the lesion appearance. A temporal

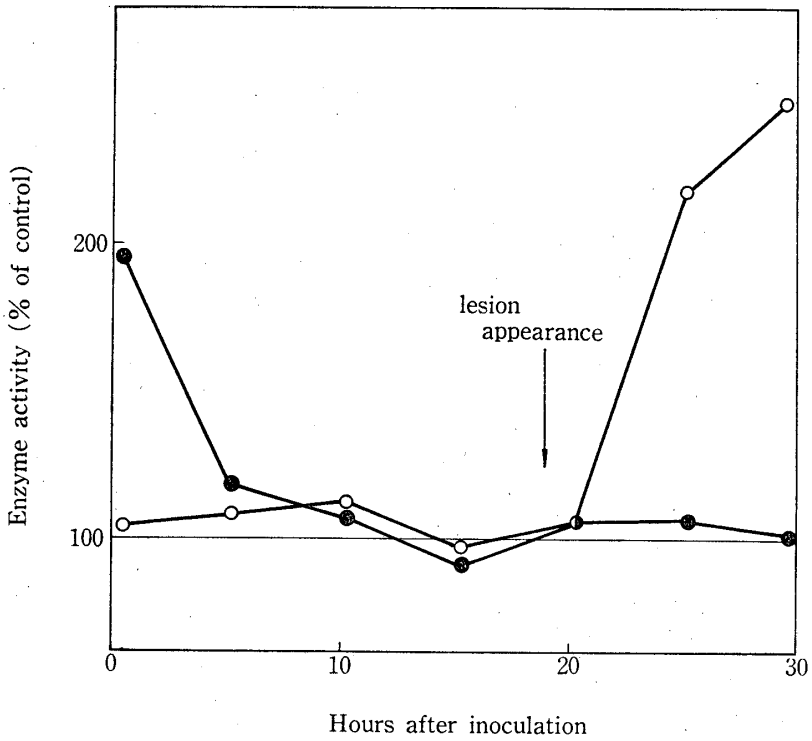

FIG. 1. Changes in activities of polyphenol oxidase and peroxidase in cowpea leaves inoculated with CMV 30 hours after inoculation. Results were expressed as per cent of control: (●) polyphenol oxidase; (○) peroxidase.

increase of polyphenol oxidase activity occurred immediately after inoculation but it returned to control level within 5 hr, perhaps due to non-specific response in the inoculated cells.

The peroxidase activity was also assayed within 30 hr after inoculation. As shown in Fig. 1, there was no increase in the peroxidase activity before the lesion became visible. However, a marked increase was found after the appearance of necrotic lesions.

Next, to gain some idea about the localization of the higher oxidizing power in the infected tissue, a benzidine solution was applied to infected cowpea leaves. A definite blue color appeared in the tissue surrounding the necrotized lesion. These results indicate that the increase of peroxidase activity is a result of necrotization.

The results for lipoxygenase activity are given in Fig. 2. Although one can observe the activation of enzyme in the period perceding lesion formation, the enzyme activity decreased more than that of the control after the lesions appeared.

On the other hand, there was no increase in phenylalanine ammonia-lyase activity before the lesion appeared. But a slight trend was apparent in the activation of the enzyme in the period after lesion formation. These results are presented in Fig. 3. It will be noted that the changes in activity of phenylalanine ammonia-layse parallel the changes in the contents of the phenolic compounds during the necrotization process.

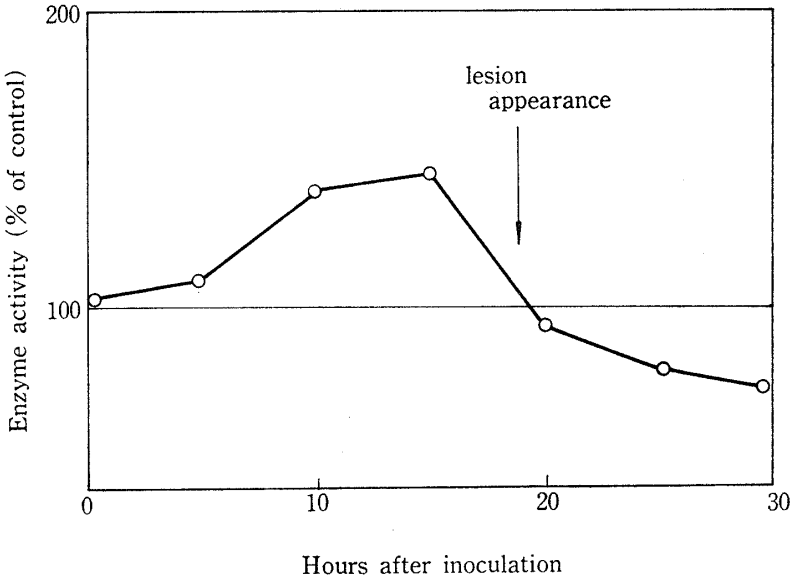

FIG. 2. Changes in lipoxygenase activity in cowpea leaves inoculated with CMV 30 hours after inoculation. Results were expressed as per cent of control.

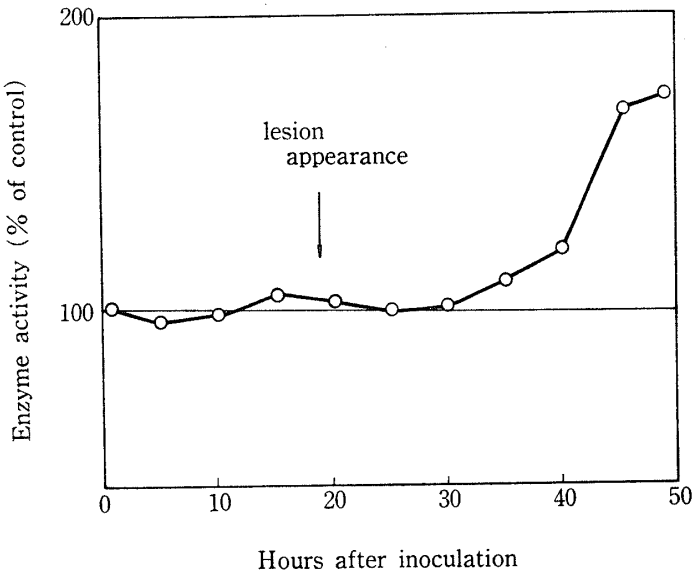

FIG. 3. Changes in phenylalanine ammonia-lyase activity in cowpea leaves inoculated with CMV 50 hours after inoculation. Results were expressed as per cent of control.

The protein content of all examined crude enzyme preparations showed no significant change throughout the experimental period.

Changes in polyphenolic compounds and flavonol contents: If the browning reaction of infected cells is accompanied by enhanced activities of polyphenol oxidase or peroxidase in the host, the concentration of substrates of these enzymes must have altered in the tissues as local lesion develops. As shown in

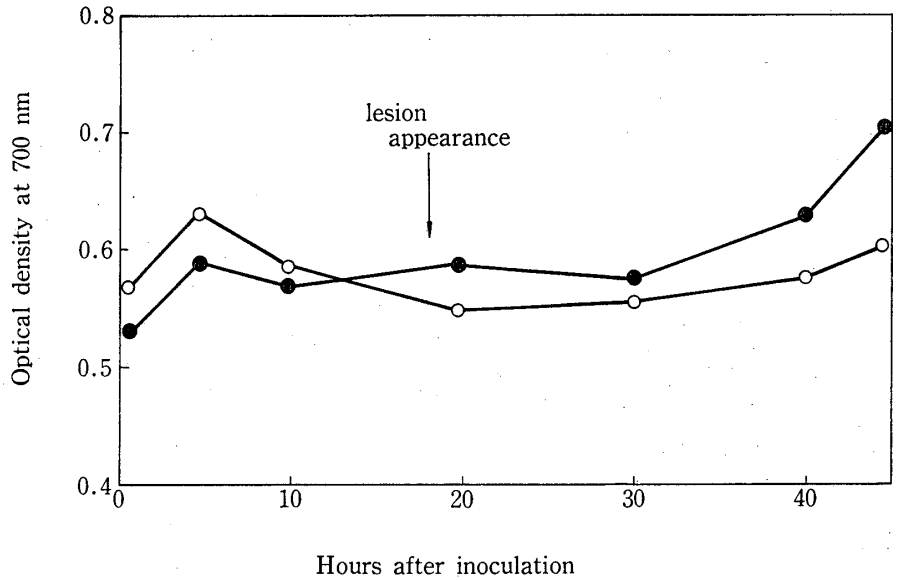

FIG. 4. Changes in the concentration of total phenolic compounds in CMV-infected and uninfected cowpea leaves. The concentration of total phenolic compounds was expressed by optical density at 700 nm: (○) uninfected leaves; (●) infected leaves.

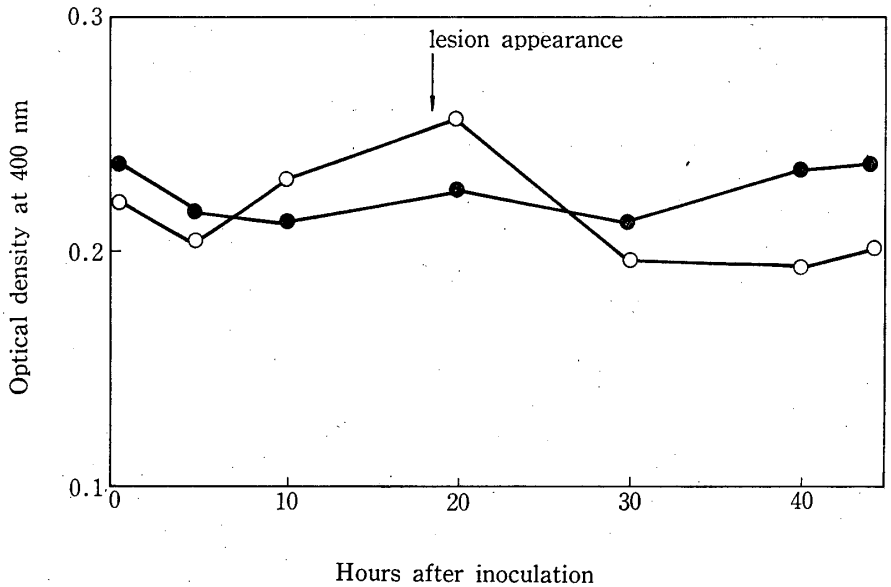

FIG. 5. Changes in the concentration of flavonols in CMV-infected and uninfected cowpea leaves. Flavonol content was calculated by substracting ε from εNa and was expressed by optical density at 400 nm. The detailed procedures are described in Materials and Methods: (○) uninfected leaves; (●) infected leaves.

Fig. 4 and 5, evidence for the decrease in their amount due to oxidation in necrotically infected leaves was not clear in this work. On the contrary, the total volume of polyphenolic compounds and flavonol showed a slight increase after the lesion appeared. Even in the cowpea leaves whose surface was 60 per cent or more

covered with coalescing necrotic lesions, the polyphenolic compounds and the flavonol contents were only slightly higher than in those of the control. However, none of the methods used here gave the expected reactions to diazotized sulfanilic acid and ferric chloride with phenolic compounds in the tissue surrounding the lesion. From these results, it is not possible to conclude that only the narrow area surrounding the lesion accumulates phenolic compounds.

Paper chromatography of phenolic compounds: The phenolic fluorescent compounds in CMV-infected cowpea leaves were examined by paper chromatography at 12 hr and 24 hr after inoculation. The Chromatograms of extracts from uninfected cowpea leaves showed the usual total of 8 fluorescent spots. No extra fluorescent metabolites were present in the infected leaf extracts at 12 hr after inoculation, but three new fluorescent spots were observed at 24 hr after inoculation.

Detection of quinone: It is well known that the quinone produced by oxidation of phenolic compounds is able to convert the leuco form of 2,3 dichlorophenolindophenol into the blue dye. Experiments were conducted to determine if the quinone was produced by the infection. Extracts of infected and uninfected tissues (boiled before extraction in 80 per cent methanol) did not turn the added leuco into blue dye. The oxidizing power of extracts did not differ significantly during lesion formation. These results indicate that there is no accumulation of quinones in the infected tissues.

Effect of enzyme inhibitors on lesion formation: If phenolic compounds are oxidized by peroxidase or polyphenol oxidase during lesion formation, browning of local lesion should be prevented by the presence of inhibitors of Cu-containing or Fe-containing enzymes. A study therefore was conducted to determine if the inhibitors of enzyme also inhibit browning of lesion. CMV-infected cowpea leaves were floated on distilled water containing NaN_3 to a concentration of 10^{-3} M. Sodium azide was chosen in the present study because this substance apparently inhibits both Cu-enzyme and Fe-enzyme, but does not inhibit lipoxygenase. The concentration of 10^{-3}M of inhibitor reduced the peroxidase activity by 85 per cent or more without affecting the lipoxygenase activity. As shown in Table 1, the visible lesion number on CMV-infected cowpea leaves was reduced about 20 per cent when NaN_3 was added immediately after inoculation. On the other hand, the formation of necrotic lesions in the treated leaves was markedly inhibited when lipoxygenase inhibitors, nordihydroguaiaretic acid (NADG, 0.05%) and gallic acid (0.1%), were applied shortly after inoculation (Table 2). However, when these inhibitors were applied after 15 hr, the lesion number was markedly reduced as compared with the control, but the size of lesions formed was very large having a diameter of about 1.5–2.0 times as much as that of the control. These results indicate that the hypersensitive reaction in processes of lesion formation is effectively inhibited by lipoxygenase inhibitors.

TABLE 1. *Effect of Sodium Azide on Local Lesion Formation of the CMV-Infected Copwea Leaf*

Concentration (Mole)	Number of local lesions	Per cent inhibition
Control	489[a]	0
10^{-1}	0	100
10^{-2}	0	100
10^{-3}	374	23.5
10^{-4}	451	7.8

a: Mean number of 15 leaves in 3 replications.

TABLE 2. *Effect of Lipoxygenase Inhibitors on Local Lesion Formation of the CMV-Infected Cowpea Leaf*

Inhibitor	Concentration (%)	Immediately[a]		15 hours	
		Number of local lesions[b]	Per cent inhibition	Number of local lesions	Per cent inhibition
Gallic acid	0	412	0	400	0
	0.01	324	21.4	307	23.3
	0.05	105[c]	74.5	319	20.3
	0.1	13	96.8	104	74.0
	0.5[d]	0	100.0	0	100.0
Nordihydro-guaiaretic acid	0	324	0	420	0
	0.01	201[c]	38.0	312	25.7
	0.05	6[c]	98.1	204	51.4
	0.1[d]	0	100.0	0	100.0

a: Time from inoculation to treatment. b: Mean number of 15 leaves in 3 replciations.
c: Appearance of local lesions in treated leaves was delayed about 6–10 hr compared with that of untreated leaves. d: This concentration produced severe phytotoxity.

Respiration: The respiration of locally infected cowpea leaves was measured periodically with a Warburg respirometer. The results are shown in Fig. 6. The respiration showed two peaks in the infected leaves. The first peak on the curve of respiration appeared at about 9 or 10 hr after inoculation and before the collapse of epidermal tissue. The second peak occurred in accordance with the time of appearance and enlargement of necrotic lesions. When inhibitors (monoiodoacetic acid and NaF) of the respiration were applied close to the time of inoculation, the respiration was markedly reduced, but no difference was observed between the inoculated and uninoculated tissues. The respiration in infected tissue was not significantly affected by inhibitors as local lesions began to appear (Table 3). These results indicate that the two peaks in the respiration curve are different in terms of sensitivity to the inhibitors of respiration. Conceivably, the initial increase of respiration may be due to the activation of the Embden-Meyerhof-Parnas pathway alone and the increase of respiration in the second peak seems to

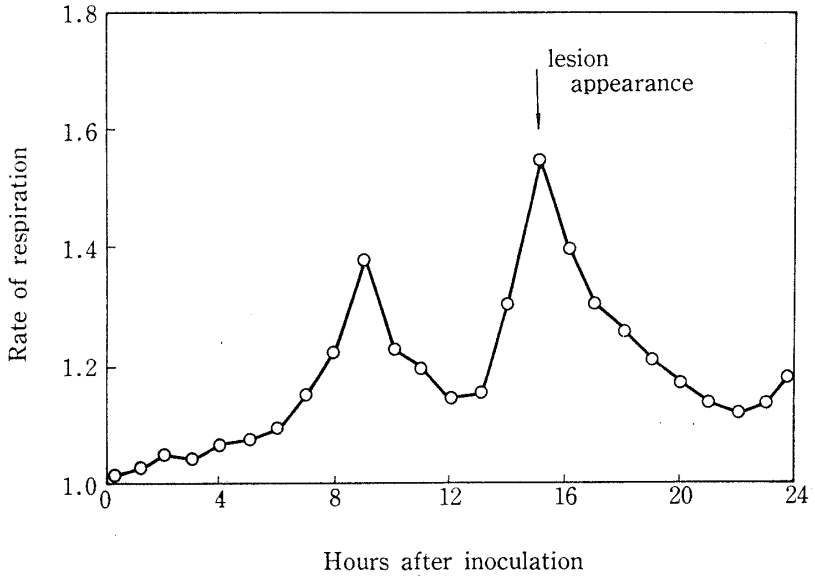

FIG. 6. Changes in respiration in cowpea leaves inoculated with CMV 24 hours after inoculation. Respiratory values were presented as the rate of infected tissue to uninfected tissue.

TABLE 3. *Effect of Respiration Inhibitors on O_2 Consumption of the CMV-Infected Cowpea Leaves*

Inhibitor	Time[a]	Per cent inhibition	
		Uninfected leaves	Infected leaves
Monoiodoacetic acid 10^{-3} M	Immediately	48.1	44.9
	15 hours after	46.2	7.3
Sodium fluoride 10^{-3} M	Immediately	29.2	26.4
	15 hours after	32.2	11.1

a: Times from inoculation to treatment.

be mainly due to the activation of the hexose monophosphate shunt.

Discussion

Ever since Szent-Györgyi and Vietorisz (20) have postulated that the phenol-polyphenol oxidase system constitutes a defense mechanism for plants, a hypothesis has been advanced that the hypersensitive reaction is caused by the accumulation of toxic oxidation products due to an enhanced polyphenol oxidase activity. Farkas *et al.* (3), working with TMV-infected *Nicotiana glutinosa*, reported similar results.

The results of the present study, however, suggest that in the case of CMV-infected cowpea leaves the polyphenol-polyphenol oxidase system is not an important factor responsbile for the hypersensitive reaction. The results presented showed that the polyphenol oxidase activity did not change before or

after the appearance of lesions, and also that there were no significant changes in amounts of phenolic compounds or in the phenylalanine ammonia-lyase and hexose monophosphate shunt activities prior to the development of a significant cytological manifestation of local lesions.

Others (17) have suggested that the increased peroxidase activity was responsible for the early killing of infected cells. In the CMV-infected cowpea leaves, however, an increase in the peroxidase activity has never been found to precede the necrosis of infected cells. Therefore, it appears likely that the increase in the peroxidase activity which occurs after the appearance of lesions is not a cause of the hypersensitive reaction, but a result of the hypersensitive death of the infected cells. Similar results were shown from histochemical work with CMV-infected cowpea leaves.

In the present study, the process of necrotic local lesion formation in CMV-infected leaves can be divided into two different stages. The initial stage is related to the hypersensitive death of the primary infected cells and the later stage is related to necrotization in the secondary infected cells surrounding the primary infected cells. The oxidation of phenolic compounds is not related to the hypersensitive reaction of the initial stage of infection, but it may be related to the subsequent necrotization as well as the enlargement of lesion size. Namely, it is considered that the oxidation of phenolic compounds is not involved in the hypersensitive reaction in the primary infected cells, because the oxidation and the accumulation of phenolic compounds does not occur before the hypersensitive reaction develops in the primary infected cells.

Since malonaldehyde, free radical intermediates and a traumatic acid like substance derived from peroxidizing lipids by lipoxygenase have been shown to increase in infected tissue*, we followed changes of both lipoxygenase activity and oxygen comsumption in the CMV-infected cowpea leaves. It was found that there was a temporal increase of both lipoxygenase activity and oxygen comsumption in the period preceding the appearance of lesion. Furthermore, the formation of lesion was significantly prevented by the inhibitors of lipoxygenase. These findings favor a possibility that hypersensitive reaction in the CMV-infected cowpea leaves is caused by the activation of some oxidative reactions in which lipoxygenase may play an important role as catalyzer. Barber and Bernheim (2) reproted that *in vitro* peroxidation of the polyunsaturated lipids found in cellular membranes led to the formation of a wide variety of toxic products capable of deteriorating cell membrane structure. Yanagida *et al.* (24) found that accompanying the peroxidation of unsaturated fatty acid, the browning of proteins occurred rapidly. On the other hand, it was thought that the traumatic acid like substance is involved in the limitation of virus multiplication or their spreading in leaf tissues, since the

* Kato, S. and Misawa, T., Unpublished data.

traumatic acid like substance isolated from CMV-infected cowpea leaves was found to have some antiviral activity. Hypersensitive reaction of CMV-infected cells is not due to the production of toxic quinone, perhaps it is due to the peroxidation of unsaturated fatty acid which brings about the breakdown of protoplasmic structure. Eventually, the cause of the hypersensitive reaction of CMV-infected cells is different from that of TMV-infected *Nicotiana glutinosa*.

Acknowledgements

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